Origin and evolution of chordates

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Origin and Evolution of Chordates

An international workshop held at Kagoshima University in Kagoshima, Japan, 2000

Preface

The following special pages in this volume are of memoirs for an international workshop on "Origin and Evolution of Chordates" organized by Drs. Hidetoshi Saiga, Hiroshi Wada and ourselves. The workshop was held on 22 and 23 November 2000 at Kagoshima University with grant supports from the Japan Society for the Promotion of Science and from the Inoue Foundation for Science.

The origin of vertebrates has been a central theme in biology and has interested comparative embryologists since the 19th century. Studies on the origin of vertebrates inevitably involve investigations of the evolution of invertebrates, especially non-chordate deuterostomes and invertebrate chordates. Alexander Kowalevsky's findings in late 19th century that ascidians and lancelets (amphioxus) develop the notochord and the latter the segmental mesoderm, dorsal nerve cord with lumen and gill slits in manners reminiscent of those in vertebrate embryos profoundly stimulated subsequent phylogenetic debates. Since then, especially during the final decade in the 20th century, with advances in molecular techniques, comparative developmental biology has undergone a renaissance and is exploring new frontiers. At the turn of the present century, paleobiology, molecular systematics, phylogenetic genomics and developmental biology are interacting to elucidate body plan evolution.

The meeting speakers include mainly developmental biologists of lancelets, and those of the ascidian, sea urchin and other non-chordate deuterostomes, phylogenetic and genomic researchers, and a Cambrian paleobiologist also joined. They were charged to interpret the body plan of deuterostomes on the basis of new findings of gene expression, experimental manipulations, anatomy, fossil records and of gene cluster duplication. They were also charged to clarify phylogenetic relationships between lancelets species in Asia and Atlantic Ocean. The meeting was the first opportunity for all of the researchers dealing with lancelet resources in their original habitats to compare their experiences. Interpretation of larval body plans can vary even on the basis of similar data: there were several examples of this at the meeting, which underscores the need for additional comparative studies of larval development.

Our hope is that this output at the workshop contained in this volume will encourage comparative research on the development of deuterostomes and be a starting point to improve our understanding of their phylogeny leading to understanding of the vertebrate origin. Finally, we express our sincere appreciation to Dr. Norio Suzuki, Editor-in-Chief of *Zoological Science* and Dr. Motonori Hoshi, the President of the Zoological Society of Japan for permitting us to publish the proceedings of this workshop.

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Chengjiang Lagerstätte and Earliest-known Chordates

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The Chengjiang Lagerstätte is located near the very beginning of the main burst of the Early Cambrian explosion (Wonderful Life by S. Gould, 1989). Great progress has recently been achieved in extensive investigations into this unique fossil treasure, with its variety of delicate soft-bodied fossils, which is one of the best windows for revealing the mystery of the Big Bang. Apart from various Ecdysozoans (Shu *et al.*, 1995a, b; Hou *et al.*, 1997) and Lophotrochozoans (Chen *et al.*, 1997; Zhang *et al.*, 2000) in Protostomia, a series of important deuterostomians, even including true ver-

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tebrates, have been unexpectedly recorded (Chen *et al*., 1995; Shu *et al*., 1996a, b; Shu *et al*., 1999a, b). Moreover, a long-awaited urochordate or tunicate, the key intermediate evolutionary form among the Chordata lineage, will be also seen soon from this treasure (Shu *et al*., in press), so that a complete early chordate evolutionary picture from Hemichordata through lower chordates (Urochordata and Cephalochordata), onto the advanced chordates (Vertebrata) from Cambrian Explosion is achieved. Here is a brief review on these discoveries.

Yunnanozoon is an enigmatic creature, which was initially put in an uncertain position. Immediately after the animal was interpreted as a possible chordate (Chen *et al*., 1995), it was reinterpreted as the earliest known hemichordate based on the evidence that it bears no convincing notochord and myomeres. However, a typical enteropneust hemichordate tripartite body-plan is able to be identified in a couple of well-preserved examples (Shu *et al*., 1996a).

Cathaymyrus has been described as an early lower chordate (Shu *et al*., 1996b). Chen *et al*. thought that the animal may be a junior synonym to *Yunnanozoon*, but without presenting any evidence (Chen *et al*., 1997). In fact, the two animals are fundamentally different from each other. First, *Cathaymyrus* bears clearly zigzag myomeres, which are further supported by its graceful snake-like or eel-like desposition, whereas *Yunnanozoon* never does. Second, the former's pharynx is restricted to its anterior one seventh area, but the structure covers the anterior one third in the latter. Third, the latter bears six pairs of gill slits only, but the former more than twenty. Lastly, The specimen of *Cathaymyrus* is brown in color, and *Yunnanozoon* is usually preserved in grey.

Xidazoon may be the most bizarre animal in the Chengjiang faunas (Shu *et al*., 1999a). Together with other enigmatic organisms (*Vetulicola*, *Pomatrum*, and other allied creatures, and possibly *Banffia*), *Xidazoon* may constitute an extinct phylum. They share a body architecture of two-fold division. The anterior division, with a big front mouth and endostyle, but without a head, represents a huge pharynx with five pairs of gill slits, and the posterior is somewhat similar to arthropods.

Myllokunmingia and *Haikouichtys* are no doubt the earliest known vertebrates (Shu *et al*., 1999b). Their mosaic characteristics (the derived characters include a large but rudimentary head, a large heart, complex zigzag-shaped myomeres, ventro-lateral paired fins and neural crest cells suggested by the gill skeleton; the plesiomorphy (primitive character state) including lacking a true backbone, retaining multiple gonads) indicates their primitiveness in the vertebrates (Shu and Chen, 2000).

The significance of these remarkable discoveries of the earliest-known chordates and their relatives from the main fountain of animals is that they may provide a direct key test to all the hypotheses on their origins, which are proposed from anatomy, and developmental and molecular biology respectively.

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On the Evolution of Chordate Body Plans

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We are interested in molecular developmental mechanisms underlying the origin and evolution of chordate body plans, which have been debated for more than a century. The notochord, dorsal hollow neural tube, pharyngeal gill slits and other morphological characters distinguish chordates from other animal groups. It is thought that chordates evolved from a common ancestor of deuterostomes (echinoderms, hemichordates and chordates) by organizing these characteristic features. Here we discuss three subjects regarding this problem.

First, we discuss an evolutionary missing link which has been debated for a long time, namely the evolution of chordate central nervous system (CNS) in relation to the larval or adult nervous system of nonchordate deuterostomes. We approached this problem by examining the expression patterns of echinoderm sea cucumber *Otx* gene and hemichordate acorn worm *Otx*, *T-brain* and *Sox B* genes. The *Otx* gene encodes homeodomain transcription factor and has a highly

conserved role in the establishment of CNS of various phyla. The sea cucumber *Otx* gene is expressed transiently in the ciliary bands of late auricularia larvae, just before metamorphosis to doliolaria larvae and the expression domain corresponded to the domains moving to the mouth during metamorphosis (Shoguchi *et al*., 2000). The acorn worm *Otx* gene is also expressed in the ciliary bands of tornalia larvae (Harada *et al*., 2000). The mammalian *T-brain* gene is expressed in the developing CNS and defines molecularly distinct domains within the cerebral cortex, and the acorn worm *T-Brain* is expressed in the tornaria larva apical organ (Tagawa *et al*., 2000), which contains the eyespots or the light-sensory organ of the larvae. The Group B *Sox* genes of vertebrates are also expressed in the developing CNS and responsible for CNS formation. We found that the hemichordate Sox genes are expressed in the ciliary bands and in the apical organ (Taguchi *et al*., unpublished). These results demonstrate that the genes responsible for chordate CNS formation are expressed in the ciliary band and apical organ of echinoderm and hemichordate larvae.

Second, we discuss our recent analysis of appendicularian (larvacean) development. Appendicularia comprises a group of pelagic tunicates that retains the tail throughout their life without exhibiting the drastic metamorphosis seen in ascidians. They possess a simple body architecture that is comparable with that of other chordates (Nishino and Satoh, 2001). We present data on cDNA and genomic clones of muscle actin (Nishino *et al*., 2000) and *Brachyury* (Nishino *et al*., 2001) of *Oikopleura longicauda*. The exon-intron organizations of these genes exhibit unconserved patterns compared with other chordates. Comparison of sequence of muscle actin isoforms indicated that the appendicularian muscle actin isoform sequence has an intermediate feature between the ascidian tail (larval) muscle actin isoform and the body-wall (adult) muscle actin isoform. The *Oikopleura Brachyury* gene is expressed in notochord cells of larvae and adults. We also discuss the structures of muscle and notochord cells in the appendicularian which suggest some interesting implications for tunicate phylogeny.

Thirdly, embryonic segmentation of amphioxus CNS is discussed. Vertebrate CNS displays the segmental pattern, which is one of outstanding feature of vertebrates. Here we show the characterization of amphioxus Hu/elav family gene (G. Satoh *et al*., unpublished). Hu/elav family genes, encoding RNA binding motifs (RRM), have been demonstrated to be involved in many different post-transcriptional events like translation, editing, transport and processing. This family gene is expressed in post-mitotic neurons and is required for its proper development. In amphioxus, the gene is expressed in individual cells of the neural plate but displays no segmental cell arrangement in early neurulae. However, in neurulae with 4 differentiated somites, segmental cell arrangement is evident, suggesting that amphioxus typically represents somite-dependent segmental body plan. We suggest here an importance of the somite for segmental cell organization of neuronal cells during amphioxus embryogenesis.

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Homeobox Genes and Axis Formation in the Ascidian Embryos

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Ascidians as well as lancelets occupy an intriguing phylogenetic position. Recent molecular phylogenetic analyses have pointed out that an ascidian embryo is likely very close to the ancestor of chordates. The ascidian embryo consists of a relatively small number of cells (the larva comprises only 2600 cells) but undergoes essentially similar morphogenesis to higher vertebrate embryos. Thus, the ascidian embryo offers a special opportunity to study a basic morphogenetic mechanism of chordate embryos. Our group has been working on the expression and function of homeobox genes of ascidians with an emphasis on axis forming and patterning mechanisms in the ascidian embryos. In the present paper, we discuss anterior-posterior patterning in the light of our findings on expression and regulation of homeobox genes in ascidian development.

Anterior-posterior patterning of the central nervous system

Our analyses of expression of homeobox genes during development of the ascidian, *Halocynthia roretzi*, demonstrated that patterning along the anterior-posterior axis of the central nervous system (CNS) exists at the level of gene expression, which is very reminiscent of the developing CNS of vertebrate embryos. The ascidian homologues of otx, *pax2/5/8* and *Hox1* are expressed in the CNS with distinct expression domains along the anterior-posterior axis (Wada *et al*., 1995). This suggests that a patterning mechanism for the CNS may be conserved between ascidians and vertebrates. In vertebrates as well as in *Drosophila*, *emx/ems* is expressed in

the anterior part of the brain like *otx/otd*. We tested whether the ascidian *emx* is expressed in the developing CNS. The ascidian *emx* was expressed in the anterior epidermis but not in the CNS (Oda and Saiga, 2001). Thus, the expression in the anterior part of the body may be old in origin. As regards absence of the expression of *emx* in the larval ascidian CNS, two scenarios are possible. Expression of *emx/ems* in the anterior CNS may be evolutionarily old and ascidians might have lost the expression in the anterior CNS. As another possible scenario, expression of *emx/ems* in the CNS may not be present in the ancestral animals but it might have been acquired independently in vertebrates and *Drosophila*. To decide between the two possibilities, further studies with other animals will be required.

Anterior-posterior patterning of epidermis

We have also demonstrated the anterior-posterior patterning is present in the epidermis of the tailbud stage embryos, in which *distalless*, *otx*, *Hox1* and *caudal* are expressed with distinct expression domains along the anterior-posterior axis (Wada and Saiga, 1999). In ascidian embryos, all of the epidermal cells are derived from the animal hemisphere. When the animal hemisphere was isolated at the 8-cell stage and cultured as an explant, none of the homeobox genes mentioned above were expressed in the explant. Taking advantage of this, we examined the effect of vegetal cells on the epidermal patterning by ablating anterior or posterior vegetal cells of the 8-cell stage embryo. From these experiments, it was concluded that anterior and posterior vegetal cells play distinct roles in anterior-posterior patterning of epidermis. We have proposed a model for a patterning mechanism of the epidermis along the anterior-posterior axis (Wada and Saiga, 1999).

Function of otx *in ascidian development*

To understand a molecular basis underlying the morphogenesis of ascidians, we have examined functions as well as expression patterns of homeobox genes. We carried out functional analysis of the ascidian *otx* gene through over-expression by injection of *in vitro* synthesized *otx* mRNA into eggs. We have found that the ascidian *otx* up regulates anterior neuroectodermal fate while it represses epidermal fate in the ectoderm. The ascidian *otx* also repressed notochord and muscle development, while it has little effect on endoderm formation (Wada *et al.*, 1999).

Transcriptional regulation of the ascidian otx *gene*

To further understand a molecular basis for the ascidian development, it is indispensable to see the transcriptional regulation of key homeobox genes. We are examining cis-regulatory elements of the ascidian *otx*. So far, we have identified a few putative elements that drive the transcription of *otx* in the anterior central nervous system (Oda *et al.*, in preparation).

Thus, ascidians offer an especially favorable system for studies of gene function and gene circuitry (Satoh *et al.*, 1996) and provide a special opportunity to study a basic morphogenetic mechanism for chordate body plan. Studies on lower chordates will facilitate understanding about a basic morphogenetic mechanism for chordates and their evolution.

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The Origin and Evolution of the Neural Crest and Insights into Evolution of the Vertebrate Face

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Traditionally, the protochordates have been believed to lack a neural crest. Neither ascidians nor amphioxus produce individually migrating cells at the boundary between the neural tube and the epidermis. However, recent evidence suggests that although they do not migrate, the epidermal cells adjacent to the neural tube are distinct from the rest of the epidermal cells.

The vertebrate *Pax3* and *Pax7* genes are expressed in both the dorsal part of the neural tube and in the neural crest, and they are essential for neural crest differentiation. The ascidian *Pax-3/7* homologue, *HrPax-37*, is comparably expressed at the early gastrula stage, not only in blastomeres fated to become the dorsal neural tube, but also in the epidermal cells adjacent to the neural plate (Wada *et al.*, 1996, 1997). These epidermal cells subsequently occupy the dorsal midline of the epidermis (dorsal epidermis). At the neurula stage, their expression is maintained only in the dorsal epidermal cells. Vertebrate *BMP4* and *BMP7* play an essential role in neural crest induction. Their ascidian homologues are also expressed in the dorsal epidermis (Miya *et al.*, 1996, 1997). Similarly, the amphioxus Dll homologue, the *AmphiDll* gene, whose vertebrate counterpart marks the neural crest, is also expressed in both the lateral edge of the neural plate, which subsequently forms dorsal neural tube cells, and in dorsal

epidermal cells (Holland *et al*., 1996). Thus, several genes involved in neural crest differentiation, are expressed in the dorsal epidermal cells in protochordates.

Sensory cells which express a sodium channel gene, *TuNaI*, are located in the dorsal midline of the larval tail. It should be noted that these sensory cells are not only located in the dorsal epidermis, but also in the ventral midline of the epidermis. Interestingly, the ascidian homologues of *Dll*, *BMP2/4*, and *BMP7* are expressed in both dorsal and ventral midline epidermal cells (Wada *et al*., 1999; Miya *et al*., 1996, 1997). This distribution of sensory cells is reminiscent of the nerve plexus of enteropneusts, which have a sensory nerve plexus in both the dorsal and ventral epidermal layers. The distribution of the ascidian sensory nerve may be an intermediate evolutionary state; only the dorsal sensory nerve remained in the vertebrates and it, together with other dorsal epidermal cells, gave rise to the neural crest.

The above evidence suggests that the dorsal epidermal cells are the origin of the neural crest. However, some neural crest marker genes, including *Snail* and *Msx*, are expressed only in the dorsal neural tube. It remains an open question whether the neural crest originated solely from the dorsal epidermis, or from both the dorsal epidermis and the dorsal neural tube of protochordates. In either case, I propose that the evolution of the neural crest is not likely to involve the birth of a new cell population, but is the acquisition of new properties by the original cell population. Examples of newly acquired cell properties are migration and the possession of anteroposterior positional information. Moreover, these two properties are key to the evolution of the vertebrate face.

Migration of the neural crest starts with segregation from the neuroepithelium. For segregation of neural crest cells, switching the expression of particular cell adhesion molecules is essential. In pre-migratory neural crest cells, *cadherin6* is expressed, while *cadherin7* is not expressed at this stage. After the onset of migration, cadherin6 is downregulated, while cadherin7 is upregulated. A small GTP-binding protein, rhoB, has a role in the delamination of neural crest cells. The molecular natures of *cadherin6*, *cadherin7*, and *rhoB* indicate that gene duplication was essential for the evolution of this system. *Cadherin6* and *cadherin7* likely evolved as a result of gene duplication in vertebrates (Wada, unpublished data). Therefore, ascidians should have a single counterpart gene of *cadherin6* and *cadherin7*, and possibly of other cadherins. A similar result is also seen in the molecular evolution of *rhoB*. Ascidians likely have one counterpart gene for the three vertebrate *rho* genes (Wada, unpublished data). These results indicate that gene duplications evolved several members of *rho* and *candherin* genes, and some of them are subsequently co-opted for delamination system of the neural crest.

Anteroposterior positional information carried by neural crest cells is essential for the patterning of facial regions. The positional information of the neural crest is encoded as *Hox* gene expression. Expression of *Hox* genes in the neural crest is, at least partially, regulated by a molecular system distinct from that used for neural tube expression. Interestingly, some amphioxus *Hox* genes regulatory elements can drive spatially localized expression in vertebrate neural crest cells, in derivatives of neurogenic placodes and in branchial arches, even though the amphioxus Hox code is restricted in the neural tube (Manzanares *et al*., 2000). This implies that the common ancestors of amphioxus and vertebrates possessed a *cis*-regulatory system of *Hox* genes which is subsequently co-opted for the neural crest specific expression, although their Hox code is operating only in the neural tube. This elaboration of *cis*-regulatory elements may have been essential for the evolution of complicated craniofacial patterning of vertebrates.

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Transcription Factors (Otx, Hox, T-brain) Involved in Sea Urchin Development

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Sea urchins are simple deuterostomes with bilaterally symmetrical, enterocoelous larvae, and are thought to possess a body plan of early deuterostomes (Davidson *et al*., 1995). *Otx*, *T-brain* and *Hox* genes, which are well conserved in metazoans, are involved in the head formation and anteroposterior positional information of segments in chordate respectively. How are these transcription factors used in the animals which have not yet evolved head and segments? In this workshop, we describe the function of these transcription

factors in sea urchin embryos.

It is well known that *Hox* gene complexes have highly conserved function in determining the anteroposterior axis. Sea urchins also possess a single *Hox* gene complex containing 10 genes (Martinez *et al*., 1999). However, in the embryo, only two *Hox* genes (*Hox7* and *Hox11/13b*) are expressed (Angerer *et al*., 1989; Dobias *et al*., 1996). The expression of *Hox7* protein is restricted to the aboral ectoderm, and *Hox11/13b* expression is restricted to oral ectoderm, endoderm and secondary mesenchyme cells in sea urchin embryos after the gastrula stage. With the aim of gaining insight into the role of *Hox7* and *Hox11/13b* in sea urchin development, we performed *Hox7* and *Hox11/13b* overexpression experiments. The overexpression of *Hox7* represses the development of oral ectoderm, endoderm and mesenchyme cells. On the contrary, overexpression of *Hox11/13b* represses the development of aboral ectoderm and primary mesenchyme cells. The data suggest that *Hox7* and *Hox11/13b* are expressed in distinct non-overlapping territories, and over expression of either one inhibits territory specific gene expression in the domain of the other. We propose that an important function of both of *Hox7* and *Hox11/13b* genes in the sea urchin embryo is to maintain specific territorial gene expression by each one, and their function does not depend on cell position along the axis of the embryo (Ishii *et al*., 1999).

Deschamps and Wijgerde (1993) reported that there are two phases of *Hox* gene expression in mouse development. The first, which is well known, takes place at the early somite stage, and the second takes place at the gastrula stage, much earlier than the somite stage. They showed that *Hox-2.3* and *Hox 2.4* start to express at the late streak stage in the allantois and the most posterior part of the streak. In *C. elegans* also, the *Hox* genes are reported to be responsible for the specification of cell fate in a position-independent manner (Wittmann *et al*., 1997; Cowing and Kenyon, 1996). The present findings in sea urchin development, and those from works in *C. elegans* and mouse early embryos suggest that the Hox gene cluster may originally have had functions in the establishment of the territories or cell-fate in a position-independent manner, and the *Hox* genes were used subsequently for determining the anteroposterior axis at the somite stage.

Otx proteins have been shown to be essential for patterning the anteriormost aspects of the brain in vertebrates. In sea urchin development, two isoforms of Otx are expressed (Mao *et al*., 1994; Sakamoto *et al*., 1997). These distinct Otx proteins are generated from a single gene by altering the transcription start site and by an alternative splicing (Kiyama *et al*., 1998). Whole-mount *in situ* hybridization using isoform-specific probes reveals a complex and dynamic change of expression patterns in the three germ layers of sea urchin embryos, suggesting that the Otx is not merely required for the differentiation of specific territories (Mitsunaga *et al*., 1998). *Otx* genes seem to be involved in the various aspects of sea urchin early development. We suggest that *Otx* may originally have had functions in the formation of all three germ layers, and the gene was used subsequently for head formation during chordate evolution.

T-brain was first isolated from the mouse. The gene was referred to as a *T-brain,* because the gene was expressed in cerebral cortex. In sea urchin embryos, *T-brain* is expressed exclusively in primary mesenchyme cells. We report that *T-brain* is involved in the differentiation and organizer activity of primary mesenchyme cells in sea urchin embryos. We suggest that *T-brain* may originally have had functions in the formation of mesoderm, and the genes were used subsequently for head formation during chordate evolution.

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New Perspectives on the Origin and Early Evolution of the Vertebrates

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In the last few years, new discoveries and advances in the areas of molecular biology, microanatomy, and paleontology have strengthened the scenario that vertebrates (used here in the broad sense to include agnathans) evolved from an ancestral invertebrate chordate. Extant invertebrate chor-

dates (namely tunicates and amphioxus), as the closest living relatives of the vertebrates, are increasingly being used as stand-ins for the proximate ancestor of the vertebrates. Each has its advantages. At present, tunicates are more favorable material for genetic study because their embryos, in comparison to those of amphioxus, are easier to work with experimentally. On the other hand, tunicates seem relatively simplified anatomically (the coeloms and posterior gut have atrophied) and genetically (divergence of *Hox* genes and loss of some of them). Therefore, it is possible that extant tunicates have lost some primitive chordate characters that are still available in amphioxus.

One of the most remarkable discoveries of the late twentieth century was the unexpected conservation of developmental gene structure and function over wide stretches of the animal kingdom. At least for animals with relatively similar overall body plans (e.g. amphioxus versus vertebrates), comparisons of the developmental expression domains of such genes have helped to evaluate body part homologies between distantly related animals (Holland and Holland, 1999). This approach has been especially useful for addressing the contentious issue of which, if any, of the major brain regions were present at the dawn of vertebrate evolution. For example, the expression domains of amphioxus genes homologous to vertebrate genes known to mark specific regions of the vertebrate brain (*Otx*, *BF1*, *Hox1*, *Hox3*, *Hox4*, and *Islet*) have been mapped onto the developing central nervous system of amphioxus. The results indicate that amphioxus, and by extension, the proximate ancestor of the vertebrates, although lacking a telencephalon, had a brain comprising a diencephalon, a mesencephalon, and a metencephalon. The identifications of amphioxus brain regions suggested by comparative developmental genetics have been well supported by recent microanatomical work based on serial transmission electron microscopy combined with computer-assisted, three dimensional brain reconstructions (Lacalli *et al*., 1994).

Expression patterns of developmental genes also provided the first hint that amphioxus and tunicates have embryonic cell populations with some similarities to vertebrate neural crest. Vertebrate neural crest cells arise at the boundary between the neural plate and epidermis, where they express a characteristic suite of genes. In amphioxus embryos, a similarly situated cell population expresses homologues of several of these neural crest-specific genes (e.g. *Dlx*, *Snail*, *Pax3/7*, and *Msx*). Vertebrate neural crest cells enter the embryonic blastocoel where they migrate and differentiate into a wide variety of cell types. In the amphioxus embryo, some of the cells at the edges of the neural plate are incorporated into the neural tube, while the adjacent epidermal cells migrate over the neural plate by means of lamellipodia. However, in contrast to the definitive neural crest cells of vertebrates, these amphioxus cells neither enter the blastocoel nor differentiate into a wide variety of cell types. Even so, despite the marked difference between these amphioxus and vertebrate cells, their developmental genetic similarities suggest that the proximate invertebrate ancestor of the vertebrates had a cell population constituting the evolutionary precursor of definitive vertebrate neural crest.

The insights gained from molecular biology and microanatomy were augmented about a year ago by the discovery of chordate fossils from the early Cambrian. These soft body fossils in the Chengjiang mudstones in south China include *Haikouichthys* and *Myllokunmingia*, which are almost certainly vertebrates, possibly related to modern lampreys (Shu *et al*., 1999) and *Yunnanozoon* and *Haikouella*, which may be stem-group vertebrates (Chen *et al*., 1999). Taken together, these remarkable new paleontological discoveries have provided several important insights into very early vertebrate evolution. For example vertebrates had an unequivocally marine origin. Moreover, very early vertebrates probably evolved mineralized pharyngeal denticles before the dermal skeleton, and evidently utilized elastic recoil of the visceral arch skeleton for suction feeding. Finally, the presence of likely neural crest derivatives (denticles and visceral arch skeleton) in the fossils gives additional support to the idea that the innovation of definitive neural crest was a supremely important event signalling the evolutionary origin of the vertebrates.

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Mitochondrial DNA Variation and Genetic Relationships of *Branchiostoma* Species from the Pacific and Atlantic Oceans

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In so far as the extant species are concerned, Cephalochordata (lancelets) represents much less diversity in terms of general body organization, species richness (represented by only about 35 known species), ecology, and reproductive biology than Urochordata and Vertebrata of the same phylum (e. g., Nishikawa and Nishida, 1997). Is the morphological uniformity among living lancelet species attributable to their recent speciation from the latest common ancestors or to the morphological stability for a long geological period since their speciation? To answer these questions, we must resolve the phylogenetic relationships among most of living lancelets. Thus, we have been conducting molecular-phylogenetic studies of them.

We compared nucleotide sequences of the cytochrome C oxidase subunit I and 16S ribosomal RNA genes on the mitochodrial genome, for two Pacific species (*Branchiostoma belcheri* (Gray, 1847), and *B. malayanum* Webb, 1955) and two Atlantic ones (*B. floridae* Hubbs, 1922 and *B. lancelolatum* (Pallas,1774)). The results were:

(1) Marked genetic differences were detected between the two Pacific and the two Atlantic species. This contrasts with the morphological information in terms of myotome number that the Pacific *B. malayanum* (51–53 myotomes) is distinct from the other three species (56-64 for *B. floridae*, 59–65 for B. lanceolatum and 62–69 for *B. belcheri*).

(2) Genetic distance between the two Atlantic species was significantly smaller than that between the Pacific pair. This result was unexpected because the Atlantic pair are located far away from each other on either side of the Atlantic Ocean (the analyzed specimens of *B. lanceolatum* coming from Roscoff and those of *B. floridae* from the Gulf of Mexico), while the Pacific pair inhabit East-Asian coasts (*B. belcheri* from Japanese and Chinese coasts and *B. malayanum* from Gulf of Thailand).

(3) Genetic distance among the analyzed Pacific and Atlantic species was calcualted 0.222 at the COI gene using Kimura's (1980) 2 parameter model. This figure was unexpectedly large for the morphological uniformity among the species, as compared with the distance (0.278) between Primates and Rodentia (sources from DNA Data Bank of Japan).

(4) Divergence time between the analyzed Pacific and Atlantic lancelets was estimated to be about 110 million years ago when Martin *et al.*'s (1992) estimate (7.0×10^{-10} transversions/site/year) from the cytochrome b and COI genes of 13 sharks was applied to the present results from the COI gene (0.078 transversions/site).

Results thus far gained suggest that the living lancelets may have far more genetic diversity than expected from the morphological uniformity mentioned above. Variety may deserve our attention even in this small animal group.

We would like to express our cordial thanks to K. Yasui and other members of the organizing committee of the "International Workshop on Origin and Evolution of Chordates" in 2000 in Kagoshima, Japan for giving us the chance to join it, and to late R. Hirakow, Y. Ojima, K. Soong, K. Shao, Y. Wang, S. Bussarawit and N. Kakhai for help and/or advice in collecting materials. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to TN (#11640694).

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The Amphioxus Genome Has Both Archetypal and Derived Features

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The new discipline of Evolution and Development (EvoDevo) tries to explain the evolution of body plans as the result of changes in developmental programs. The general rule of EvoDevo research is that most genes and gene networks are conserved among most animal phyla and play similar roles during development of very different animals. However, the paradox of EvoDevo is that animals truly evolve distinct morphologies using similar *pieces* (or genes) and *kits* (genetic networks). One of the possible solutions to this paradox is gene duplication, co-option, and evolution of cis-regulatory elements.

The invertebrate/vertebrate transition is a critical landmark from the EvoDevo point of view: several new features were invented or increased markedly in complexity. In particular, evolution of the vertebrates involved the origin of new cell types and organs as well as increased body organization complexity. This transition was most probably linked to events of gene duplication (Holland *et al.*, 1994). As human and other vertebrate genome mapping and sequencing advances, it is becoming clearer that the vertebrate genome results from extensive gene duplication events.

Amphioxus (*Branchisotoma*, Cephalochordata), are thought to be the closest living sister group relative to the vertebrates (Wada and Satoh, 1994), retaining many archetypal features of the simpler vertebrate-like body plan without showing major complications. In recent years, most examples of genes and gene clusters identified in amphioxus show that its genome also reflects this archetypal organization. In particular, amphioxus has a single Hox cluster (Garcia-Fernàndez and Holland, 1994) whereas higher vertebrates possess at least four. The single amphioxus Hox cluster and the one-per-class gene identified in most amphioxus-oriented labs (see this issue) have placed amphioxus at the privileged position of being considered "the" ancestor of vertebrates, thus retaining a "frozen" ancestral genome and an ancestral body plan. Furthermore, the expression pattern of developmental genes in amphioxus has been considered equivalent to the ancestral function of such genes at the origin of vertebrates. However, amphioxus, itself, has evolved since the divergence from the ancestor of vertebrates, more than half a billion years, and its genome and gene functions should reflect such history, including divergence and particular oddities.

Most examples of gene evolution in the amphioxus lineage reflect the side of conservation: the finding of the ParaHox cluster, the evolutionary sister of the Hox gene cluster, was even predictive to other genomes (Brooke *et al.,* 1998); the expression pattern of amphioxus genes *Hox-1*, *Hox-3* and *Hox-4* (Wada *et al*., 1999) revealed cryptic segmentation in the amphioxus nerve cord, revealed also the homology between the vertebrate hindbrain and an extensive region of the amphioxus neural tube, and suggested the ancestral function of the chordate Hox cluster in the regionalization of the neural tube.

However, several other examples reflect the evolved side of the amphioxus genome. As an example, *AmphiHox-2* has lost its role as a cannonical *Hox* gene, and is no longer expressed in the neural tube in a collinear manner. This may reflect a secondary modification or simplification of the most anterior part of the amphioxus neural tube, and it is correlated with a higher degree of divergence with respect to vertebrate group 2 genes.

In addition, we have performed a chromosomal walk at the posterior end of the amphioxus Hox cluster. We found 4 new *Hox* genes in approximately 100Kb upsteream of *Hox-10*. We called these genes *AmphiHox-11*, *–12, –13* and *–14*, depending on their chromosomal position (Ferrier *et al*., 2000). We have further continued the walking for 40 Kb upstream *Hox-14* but no other Hox gene (*Hox 15*?) has been detected (Minguillón and Garcia-Fernàndez, unpublished). Remarkably, amphioxus is the first animal in which a *Hox 14* gene has been found, and possesses the most gene-rich Hox cluster to date. It seems apparent that the posterior end of the amphioxus Hox gene cluster is not as archetypal as the anterior and central parts of the cluster. Molecular phylogenetic analyses in order to investigate the relationships between chordate posterior genes did not resolve whether the amphioxus posterior genes are orthologous (pro-orthologous) to vertebrate posterior genes, or arose after independent duplication events in the amphioxus lineage. We propose that the lack of resolution of chordate (and deuterostome) posterior genes is due to a higher degree of evolution, compared to anterior genes. The faster evolutionary rate of posterior genes may be due to the phenomenon of posterior flexibility, which reflects a higher constraint of anterior genes to evolve. Posterior flexibility would obscure the pro-orthologous character of amphioxus with respect to vertebrate genes. Intriguingly, *AmphiHox-14* remains a candidate for a duplication in the amphioxus lineage (an oddity of amphioxus genome) and a candidate for a new archetypal gene, thus implying the existence of such group within vertebrates, a 14th Hox group that has not yet been found.

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An Amphioxus Gene Catalogue

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Besides the human genome, most genomic or EST sequencing projects are focused on two groups of organisms: the ecdysozoans (represented by *C.elegans* and *Drosophila*) and three vertebrate deuterostomes; mouse, zebrafish and *Xenopus.*

These two groups have evolved separately for 550 Ma during which period, two whole genome or tandem gene duplications have probably occurred on the vertebrate lineage resulting in vertebrates provided with perhaps four times more genes than invertebrates. As a consequence of the gene duplications and independent lineage evolution, there is limited sequence conservation between the above two animal groups, making it difficult to recognise homologous genes, to distinguish between orthologs and paralogs and to correlate

the functional information generated.

Finally, the two groups differ significantly in their adult morphology and mode of development.

Which genes are present in the invertebrate ancestor of the vertebrates? What is the structure of these genes as compared to the ecdysozoan or vertebrate sequences?

We answer these questions through the amphioxus genes. Amphioxus, a cephalochordate separated last from the chordate lineage leading to vertebrates. We have used the oligonucleotide fingerprinting (Clark *et al.*, 1999) normalisation method to create a non-redundant catalogue of transcripts expressed at the gastrula (5–6 hr) and neurula (26 hr) stages of amphioxus (*Branchiostoma floridae*) and estimate the number and the relative expression level of genes present at the above two stages. 5' ESTs of 14,000 representative clones from both libraries and their subsequent clustering in consensus sequences confirmed the efficieny of oligonucleotide fingerprinting method in reducing redundancy.

The identified amphioxus transcripts range from genes controlling metabolism and gene/protein expression, to cell signalling and cell communication molecules. The presence of known domains was detected in all sequences and their frequency was compared with those in invertebrate proteins and all vertebrate publicly available Unigene sequences. We found examples supporting the view of two gene duplications after the separation of cephalochordates such as the amphioxus homolog of the vertebrate bone morphogenetic proteins BMP2 and BMP4 (AmphiBMP2/4) (Panopoulou *et al.*, 1998), as well as gene families that have evolved at a rapid rate in the amphioxus lineage such as members of the zinc finger gene family (unpublished) or intermediate filament proteins (Karabinos *et al.*, 2000) and homologs of the Wnt gene family (unpublished).

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Cloning and Embryonic Expression of *Bblim3*, a LIM Homeobox Gene in Amphioxus (*Branchiostoma belcheri tsingtauense*)

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The vertebrate body plan is established by a series of inductive interactions and cellular rearrangements. Cellular and molecular mechanisms underlying these processes are perhaps best understood for amphibia. There are two organization centers in the early developmental process: the Nieuwkoop center and Spemann's organizer. After the mid-blastula transition, zygotic gene products from the Nieuwkoop center induce Spemann's organizer in the overlying equatorial region.

Spemann's organizer is a region of the amphibian gastrula that is capable of inducing a second body axis upon transplantation to ventral or lateral regions of a host embryo. Spemann's organizer provides signals that dorsalize mesoderm, induce convergent-extension movements in ectoderm and mesoderm and specify neuroectoderm. Functional equivalents of the amphibian organizer have been identified in other vertebrates, for example, the embryonic shield in zebrafish and Hensen's node in amniotes. However, the origin of Spemann's organizer is unclear. Therefore, it is necessary to know whether there is a functional equivalent of the amphibian organizer in lower chordates or not. Based on this consideration, we plan to study some genes related to the vertebrate organizer. These genes include LIM homeobox genes, *Otx, Nodal, Xnot,* etc. Amphioxus *lim3* is the first one we have studied.

Bblim3 cDNA was isolated from a neurula cDNA library of amphioxus (*Branchiostoma belcheri tsingtauense*). This gene encodes a typical LIM-type homeodomain protein that contains two tandemly repeated LIM domains and the homeodomain. Similarities of the predicted amino acid sequences between amphioxus *Bblim3* and other known *lim3* genes are very high in the homeodomain, all of which are higher than 90%. Also, the two LIM domains are well conserved (>60%). Based on the alignment among full length sequences of known *lim3* genes, we have constructed a phylogenetic tree, which shows *Bblim3* has a closer relationship with vertebrate *lim3* genes than *lim3* homologues of other invertebrates.

We utilized whole mount *in situ* hybridization method to reveal the expression pattern of *Bblim3* in developing embryos. Signals were first detected in the gastrula, and expression continued at least until the 40 hr larvae. In the gastrula, the transcripts were detected in the ventral endoderm. In the neurula, the transcripts were detected in the neural tube and the tissue close to the ventral neural tube. In 40 hr larvae, transcripts were detected in Hatschek's pit, the anterior region of

neural tube and in a region close to the tail tip.

Vertebrate *lim3* is expressed in discrete subtypes of motor neurons and in the anterior and intermediate lobes of the pituitary. Hatschek's pit is regarded as a homologue of the pituitary gland in vertebrates. Our result gives support for this view. However, the expression of *Bblim3* in gastrula is quite different from that of vertebrate *lim3* genes.

Previously, we have discovered that ascidian homologues of *lim1* and *lim3* show quite different relation between their sequences and the expression patterns from that of the vertebrate *lim1* and *lim3* genes. *Hrlim2*, the ascidian homologue of vertebrate *lim1*, shows a similar expression pattern to that of vertebrate *lim3* (Saiga *et al*., in preparation), while Hrlim, the ascidian homologue of vertebrate *lim3*, shows a similar expression pattern to that of vertebrate *lim1* (Wada *et al*., 1995). As we reported above, the expression pattern of *Bblim3* is quite different from that of vertebrate *lim3*. Thus to elucidate the expression pattern of the amphioxus *lim1* homologue should be intriguing.

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Specificities of the Early Body Formation in the Lancelet Embryo

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Lancelet development and anatomy show many characters comparable to those of vertebrates, and thus the animal is of great interest for the study of the chordate phylogeny. However, paleobiological studies are supplying evidence that most extant groups of the deuterostomes had emerged by Lower Cambrian (Shu *et al*., 1999). Molecular phylogenetic studies also tell that it is rather difficult to gain a reliable phylogram for chordates from molecular data (e.g., Naylor and Brown, 1998). In this situation, it is important to clarify what character in the lancelet development is similar to (if possible, shared with) and is different (derived) from the vertebrates. Here we offer two developmental characters of a Chinese lancelet, which make us reconsider the phylogenetic relationship of lancelets.

We first examined expression pattern of genes encoding secreted proteins, BbWnt7, BbWnt8 and BbBMP2/4, as well as transcription factors, *brachyury*, *hnf-3* class and *T-br* genes (probes for *brachyury* and *hnf-3* mRNAs were gifted from Drs. PWH Holland and SM Shimeld, respectively). These genes are expressed in early development and considered to play important roles in early body formation in vertebrates. All of these genes in lancelets except for *Bbbmp2/4* were expressed in similar fashion to those of the vertebrates. *Bbbmp2/4* was expressed in the mid-dorsal inner layer of gastrulae and widely in the anterior region, in which BMP antagonists such as Chordin, Noggin and Cerberus block the signalling in vertebrates. Since embryonic origin of the anterior specific structures, such as anteriorly extended notochord, organs derived from the lateral diverticula and the lateral diverticulum itself, corresponds to the anterior domain where *Bbbmp2/4* was continuously expressed, we think that BMP signalling may play a key role in developing the specificity. Furthermore, the expression pattern of these genes showed that the lancelet embryo has two distinct developmental domains from the gastrula stage (Yasui *et al*., in press). Lancelet *hnf-3* and *Bbbmp2/4* were expressed anteriorly and *brachyury* and *Bbwnt8* were expressed in the posterior half at the mid-gastrula stage. Slightly later a *T-br* gene was expressed in the anterior region of the archenteron. Although the anterior genes modify their expression pattern, the posterior genes continued to be expressed within the region where the somitocoelomic system is differentiating. *Bbwnt7* , which was expressed within the CNS, also showed an anterior boundary of expression approximately coincident with the anterior margin of the somitocoelomic system. The two distinct domains found in this study seem to correspond anatomically to the protosome and metasome, respectively, in the deuterostome tripartite body plan. However, since *hnf-3β* and *eomesodermin* (*T-br2*) are expressed in the anterior primitive endoderm in the mouse, it may be possible that the dichotomous developmental pattern in lancelets is a plesiomorphy of the deuterostomes. For further discussion, we need related data from non-chordate deuterostomes.

The second observation is distribution of β-catenin proteins in early embryos. Immunostaining against β-catenin with an anti-β-catenin antiserum (C2206, Sigma, USA) showed that cytoplasmic β-catenin was unevenly inherited by the blastomeres during the first cleavage. The uneven distribution was recognizable at least until the 32-cell stage. Nuclear translocation was detected at the 64-cell stage, and at the blastula stage all cells showed the nuclear accumulation. During gastrulation, nuclear β-catenin was observed in the dorsal region as in vertebrates. Although distribution of β-catenin in blastulae and gastrulae suggests a similar function of this molecule to the vertebrate counterpart, that in early embryos is curious. The asymmetric distribution at the 2-cell stage indicates that

the dorsoventral polarity is established during the first cleavage in lancelets. To examine this possibility, we separated the blastomeres at the 2-cell stage and checked their development. Of 17 manipulations, 12 pairs showed one normal embryo and the other abnormal or dead embryo. The result seems to support the asymmetric division of the first cleavage, although we need more samples to confirm statistically.

We also examined perturbation of β-catenin behavior by LiCl. Embryos treated with 50 mM or 100 mM LiCl from one-cell to 128-cell stage showed a variety of phenotypes. The cleavage pattern of many embryos was affected and later many showed uncleaved mass with small blastomeres being immunoreactive with the anti-β-catenin antiserum. Most embryos freed from the abnormal cleavage seem to stop development at the mid-gastrula stage and remained with a widely open blastopore. Moderately affected embryos were able to elongate anteriorly, still showing a widely open blastopore and defects in notochord formation. Some embryos resulted in exogastrula-like phenotype. Interestingly, LiCl-treated 2-cell embryos showed evenly distributed cytoplasmic β-catenin and seemingly abnormal cleavage. The observation suggests a possibility that lancelets may uniquely establish the dorsoventral polarity by the asymmetric first cell division, and that this phenomenon is susceptible to LiCl.

The two distinct developmental domains in embryos and a possible asymmetric distribution of β-catenin at the 2-cell stage in lancelets are inconsistent with the idea that the cephalochordate is a sister group of the vertebrate. In the Cambrian period, there were curious animals that possess body plans seemingly mixed with those of present-day animals (Gould, 1989; Shu *et al*., in this issue). Phylogenetic reconstruction based on nucleotide and amino acid sequences does not lead to consistent results, which is considered to be due to defects in methodology or nature of data, in general. However, it might reflect the real history of evolution. More intensive comparative study of developing embryos is needed over a wide spectrum of animal groups.

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Amphioxus and the Evolutionary Conservation of Anterior-posterior Patterning

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Among the deuterostomes, radial, indeterminate cleavage and eggs with little yolk are considered primitive. In echinoderms and amphioxus, gastrulation is by invagination and the vegetal pole of the egg approximately corresponds to the posterior pole of the embryo. Development in tunicates is quite modified with early determination of cell fates, a non-feeding larva lacking mouth and anus and a hindgut reduced to an "endodermal strand." In tunicates, the vetgetal pole/gastrulation site is skewed to the posterior-dorsal side. In addition, elongation of the tail does not involve growth from the tailbud as in other chordates. In vertebrates, gastrulation, modified by the acquisition of considerable yolk and/or extraembryonic membranes, is by involution. In spite of differences in gastrulation, early expression of *Notch*, several *Wnt* genes and *brachyury* in the cephalochordate amphioxus is very similar to the patterns of their homologs in echinoderms and vertebrates. These genes are early markers for presumptive mesoderm, being first expressed in a ring of mesendoderm just inside the blastopore of the gastrula. Subsequently, they are expressed in the posterior mesoderm. *Brachyury* is the first to be expressed in the future blastoporal lip (Holland *et al*., 1995; Zhang *et al.,* 1997). *Wnt8* turns on about the same time (Schubert *et al*., 2000a; M. Schubert, pers com). Followed by Wnt1 (Holland *et al*., 2000) and then by *Notch* (Holland *et al*., 2001), and finally *Wnts 4*, *7b* and *11* (Schubert 2000b, 2000c). In the late gastrula and neurula, Wnt1 remains expressed around the blastopore. Later, expression of *Notch*, *brachyury* and some *Wnt* genes (i.e. *Wnts 4*, *8*, *11*) expands into the somites, notochord and neural plate. The spatiotemporal expression of these genes in amphioxus suggests that the *Notch* and wnt/wingless pathways and *brachyury* may act together in patterning the mesendoderm.

Homologs of *brachyury*, *Notch* and genes in the wnt-signaling pathway are expressed in sea urchins in similar patterns as in amphioxus. Although sea urchin embryos form neither somites, a notochord or nerve cord, there is a posterior/vegetal Notch and *Wnt* signaling center in the early embryo. In sea urchins, the vegetal pole of the late blastula flattens to form the vegetal plate, which gives rist to the mesoderm and invaginates to form the embryonic gut. Notch protein becomes localized to the apical surfaces of cells at the edges of the vegetal plate, later becoming localized to the apical surfaces of cells around the blastopore and in the invaginating endoderm (Sherwood and McClay 1997, 1999; Sweet *et al*., 1999). This pattern is similar to that of *Notch* expression in the amphioxus gastrula. Similarly, *Wnt8* and β-catenin become localized to the vegetal region of the sea urchin embryo. In sea urchins, experimental evidence shows that wnt-signaling patterns along the anterior/posterior (animal/vegetal) axis and that the wnt and Notch pathways interact. *Brachyury* is also expressed in the sea urchin vegetal plate. In starfish, but not sea urchins, it is subsequently expressed around the blastopore.

In the embryos of several species, the Notch and Wnt pathways have been shown to interact at several levels and the transcription factor *brachyury* to be a downstream target of both pathways (reviewed in Panin and Irvine, 1998; Dierick and Bejosovec, 1999). Typically, the Notch and Wnt pathways play opposing roles in developing tissues (Brennan *et al.*, 1999; Uyttendaele *et al.*, 1998). The secreted protein wnt/wingless can act as a ligand of the transmembrane protein Notch, binding to its extracellular domain, but it can also affect Notch signaling through interaction of dishevelled, a down-stream component of the wnt-signaling pathway, with the intracellular domain of Notch (Wesley and Saez, 2000; Axlerod *et al.*, 1996). Conversely, Notch signaling can regulate wnt/wingless (Panin and Irvine, 1998).

In tunicates, the Notch pathway has been shown to be upstream of *brachyury* (Corbo *et al.*, 1997, 1998), while in *Xenopus*, the *brachyury* promoter binds the downstream component of the wnt-signaling pathway, LEF-1 (Arnold *et al.*, 2000).

In vertebrates, *Notch*, *brachyury* and several wnt genes are similarly expressed in the posterior mesendoderm. Although the wnt-signaling pathway first acts in *Xenopus* to establish dorso-ventral polarity of the blastula, a second phase of wnt-signaling is involved in posteriorization of the neuroectoderm, formation of paraxial mesoderm and tailbud extension. Our results suggest that a similar mechanism for patterning along the early animal/vegetal (anterior-posterior) axis involving interaction of the Wnt and Notch pathways and *brachyury* acts in amphioxus and, by extension, was present in the ancestral deuterostome.

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Axial Signalling and the Evolution of Dorsoventral Pattern in Neurectoderm and Mesoderm

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During vertebrate embryogenesis both neurectoderm and mesoderm develop complex dorsoventral patterns of cell types, typically reflected in mirror image around the embryonic midline. The notochord and floor plate play a key role in controlling the development of this pattern, which is in part mediated by hedgehog signalling from these midline structures. Experimental evidence suggests that hedgehog signalling induces the formation of ventral neural and medial mesodermal cell types, and works in competition with Bmp signalling from ectoderm and lateral plate mesoderm. The invertebrate chordate amphioxus also develops both molecular and morphological dorsoventral pattern of the neural tube and mesoderm (Holland *et al.*, 1995; Shimeld, 1999a). Previously (Shimeld, 1999b), I have shown that an amphioxus *hedgehog* orthologue is expressed by both neural and mesodermal midline cells, suggesting that the pathway that patterns the dorsoventral axis of the neural tube and mesoderm is shared between amphioxus and vertebrates and therefore predates the evolutionary origin of vertebrates. Most, and possibly all, hedgehog signalling feeds through the protein products of the *Gli* genes, a family of C2H2 zinc finger transcription factors. A closely related family of C2H2 zinc finger transcription factors, the *Zic* genes, may modulate Gli function and Gli interpretation of hedgehog signalling. Both families are represented by multiple genes in vertebrates. In amphioxus, reciprocal Southern blotting shows that both families are represented by single genes, and therefore that the multigene families found in vertebrates are due to duplications specific to the vertebrate lineage. Vertebrate *Zic* and *Gli* genes are expressed in specific patterns in the developing nervous system. *Zic* gene expression marks areas where neurogenesis does not occur, including the neural crest, and in overexpression assays *Zic* genes are strong inducers of neural crest and inhibitors of neurogenesis (Brewster *et al.*, 1998; Kuo *et al.*, 1998; Mizuseki *et al.*, 1998; Nakata *et al.*, 1998). In contrast, *Gli* genes seem to act in an antagonistic manner to *Zic* genes and promote neurogenesis. In amphioxus the spatial and temporal expres-

sion of *Zic* and *Gli* generally resembles that of the vertebrate family members. A potentially important difference is that in amphioxus *Zic* expression is lost from the lateral neural plate at the end of neurulation, and is only re-established after neural tube closure. Therefore, during a developmental period when, in vertebrates, neural crest specification is underway, amphioxus lacks expression of a potent neural crest inducer but retains expression of antagonistically-acting Gli expression. This may have implications for understanding some of the molecular changes behind the origin of the neural crest.

Amphioxus *Gli* and *Zic* genes are also expressed in the developing paraxial mesoderm of the amphioxus neurula and post-neurula. Previously Holland *et al.* (1995) have noted three mesodermal compartments in amphioxus; a medial compartment which forms the myotome which is such a prominent feature of the adult amphioxus, a ventrolateral compartment which forms the coelomic cavity surrounding the gut and a dorsoventral compartment which forms the coelomic cavity surrounding the notochord. Amphioxus *Gli* and *Zic* expression respects these various compartment boundaries, suggesting that their development is influenced by hedgehog signalling from the notochord. Specifically, Zic expression is confined to the dorsolateral compartment. It is notable that in vertebrate development, somitic Zic expression is confined to a topographically similar portion of the somite, suggesting that, in common with the neural tube, some aspects of the molecular patterning and subdivision of the paraxial mesoderm represent ancestral characters shared between amphioxus and vertebrates.

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Left-right Asymmetric Oral Innervation Involving the Oral Nerve Ring in Lancelets

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Lancelets (amphioxus) are the only animal in which left-right asymmetry is conspicuous in the larva and then becomes much more symmetric during later development. However, traces of the left-right asymmetry are found at adulthood. The oral innervation is an example in which not all nerves but only left nerves 3 to 5 (nerves 3L–5L) show an asymmetric pattern (Franz, 1923). Nerves 3L and 4L extend contralateral branches to the oral hood or the velum crossing ventral to the notochord. To investigate the formation of the asymmetric innervation found in specific lancelet nerves, the peripheral nerves in the anterior region were studied in pre- and mid-metamorphic larvae, 1-cm-long juveniles, and adults by using wholemount immunostaining technique.

At the early pre-metamorphic larval stage, nerve 2L left the oral region (Yasui *et al.*, 1998), and nerves 3L–5L innervated the region. At the late pre-metamorphic larval stage, the mouth had expanded posteriorly on the left side, and thus nerves 6L and 7L came to participate in the innervation of the oral region. In 27-day-old larvae, an intrinsic nervous system called the oral nerve ring (Lacalli *et al.*, 1999) had been formed around the mouth, with which nerves 3L-6L from the CNS connected. The nerve ring in 27-day-old larvae extended a thick branch anteriorly from the antero-dorsal part of the ring portion. We designate the branch portion and the ring portion as the pre-buccal branch and the secondary oral nerve ring, respectively. The pre-buccal branch reached half way between the preoral pit and the mouth. Nerve 3L connected with the tip of the branch and nerve 4L with the junction between the branch portion and the ring portion. Nerves 5L and 6L reached the dorsal part of the ring portion.

At the initial stage of metamorphosis, the integumental fold (Willey, 1891), the precursor of the left oral hood, appeared along the dorsal margin of the mouth, and between the mouth and the preoral pit, the pre-buccal cavity (Stokes and Holland, 1995) was formed in accordance with the posterior shift of the mouth. Nerve 8L came to participate in the innervation of the oral region. Nerves 3L–6L maintained their connection with the oral nerve ring, but the connection pattern became rather complicated. The pre-buccal branch elongated further anteriorly to reach dorsal to the preoral pit, while nerve 3L kept on connecting with its anterior tip. The pre-buccal branch is the precursor of the left part of the inner oral-hood nerve plexus (see below). Later, nerve 3L extended a ventral branch from the junction, which passed through the anterior margin of the preoral pit. Nerve 4L also kept the connection with the pre-buccal branch and grew further ventrally to connect with the anterior part of the secondary oral nerve ring. Nerves 5L and 6L connected with the dorsal part of the

secondary oral nerve ring as before. A thin nerve plexus apart from the secondary oral nerve ring was formed at the posterodorsal margin of the mouth. This plexus seems to be the developing outer oral-hood nerve plexus (see below). It received fibers from nerves 7L and 8L.

The 1-cm-long juveniles had developed the oral hoods, and the mouth had been shifted to the mid ventral region as in adults. Two nerve plexuses were formed at the margin of the oral hoods. One is designated the inner oral-hood nerve plexus and the other the outer oral-hood nerve plexus. The inner plexus was asymmetrically connected only with nerves 3L–5L on both sides, whereas the outer plexus received nerves 3–7 ipsilaterally and segmentally on either side. The inner oral-hood nerve plexus completely encircled the distal margin of the oral hoods, anteriorly by the contralateral branch of nerve 3L and posteriorly by the nerve plexus itself. The configuration of the inner oral-hood nerve plexus suggests that it is derived from the oral nerve ring. The innervation to the velum had also been complete in the juveniles, in which branches from nerves 4L and 5L were involved. The velar innervation and the translocation of the larval mouth (Stokes and Holland, 1995) suggest that the velar nerve ring is also derived from the secondary oral nerve ring. Nerve 6L had left the inner oral-hood nerve plexus.

The present observations show that the anterior peripheral nerves in lancelets except for nerves 3L–5L change their innervation patterns during development and that the basic pattern of the oral innervation including the asymmetric pattern appears at the early metamorphic larval stage. Furthermore, it is suggested that the oral nerve ring is the precursor of both the inner oral-hood nerve plexus and the velar nerve ring and that the asymmetric innervation in adults results from the persistent connection between nerves 3L–5L and the oral nerve ring in pre-metamorphic larvae.

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Muscle-related Genes and Immune-related Genes Derived from Amphioxus Notochord cDNA Library

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The notochord is a defining character of chordates and the elucidation of its development and function is central for understanding the evolution of chordates. However, little is known about what kinds of gene are expressed in the amphioxus (the subphylum Cephalochordata) notochord during its differentiation, except that *Brachyury* is expressed there. The notochord of amphioxus runs the full length of the midline of the body extending from the anterior most part of the rostrum to the tip of the tail, and morphological studies have shown that it contains myofilaments in the cytoplasm. The present EST (expressed sequence tag) analysis targeted mRNAs of the amphioxus notochord to determine genes that are expressed there (Suzuki and Satoh, 2000). Notochord cells were isolated from *Branchiostoma belcheri* adults, from which a cDNA library was constructed. Blast search of a set of 257 ESTs revealed that about a half of the clones showed significant similarities to known proteins. They included muscle related genes and immune-related genes.

Muscle-related genes comprise about 11% of the clones, which represent 12 different types of genes. One of them was a novel notochord-specific actin gene, of which the amino acid sequence is distinct from cytoplasmic actins and muscle actins. We then investigated the expression pattern of these muscle-related genes during development. The 12 genes were categorized into two groups; (1) genes specifically expressed in the notochord from the neurula stage when the notochord is formed, and (2) genes first expressed in somites in the neurula and then expressed in the notochord in early larva when the striated structure appears in the notochord. As far as we know, these muscle-related genes are not expressed in the notochord of ascidians (the subphylum Urochordata) or vertebrates. We suppose that transcription factors that control the muscle related gene expression came under the control of the master regulators of the notochord such as *Brachyury* only during evolution leading to cephalochordata.

In addition, the EST clones contained six different genes that are involved in the immune system. These correspond to 6% of the EST clones. Among them, we found two complement components: AmphiC3 and AmphiC6. AmphiC3 is a homolog of C3 of vertebrates, ascidians and sea urchin, that plays a central role in the complement system. AmphiC3 retains all basic structural characters of mammalian C3. AmphiC6 is one of the terminal components, C6, C7, C8 and C9, which function in a cytolytic pathway. Because the terminal components have been considered to be present only in jawed vertebrates, this is the first report of an invertebrate C6, suggesting an ancient origin of the cytolytic complement system during chordate evolution. Phylogenetic analysis with a neighber-joining tree showed AmphiC6 forms a cluster with human C6 that is supported by a high bootstrap percentage.

It is suggested that gene duplication among the terminal components occurred before the divergence of cephalochordata from vertebrates. *In situ* hybridization showed that the transcript of *amphiC3* is specific to epipharyngeal groove and hepatic cecum, while *amphiC6* is expressed only in epipharyngeal groove. Both the hepatic cecum and epipharyngeal groove are secretory organs. These complement components are thought to work in the pharynx and break down bacterial food.

The other four genes are related to innate immunity. One is a *defensin* gene which encodes a protein having a strong antibacterial activity. *In situ* hybridization showed that amphioxus *defensin* is expressed only in the notochord cells from larval stage to adulthood. It shows a possibility that the amphioxus notochord has a function in immune defence system.

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Hatschek's Pit: Its Endocrinological Significance

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Amphioxus has been considered to be an endangered animal in Japan. Available populations have gradually declined, the collection of benthic amphioxus is not easy, and it has been virtually impossible to obtain fertilized eggs in Japan. Recently, large populations were discovered in Amakusa area, Kyushu Dist., Gokasho Bay, Mie Pref. and Enshu-Nada Sea, Aichi Pref. Since 1996, ecological surveys of the amphioxus population in Enshu-Nada Sea have been periodically carried out by using the research vehicle, Tansei-Maru, of the Ocean Research Institute, University of Tokyo (Kubokawa *et al*., 1999). Populations in two areas, located at 10 km off the southern coast of Atsumi Peninsula and in the shallows near Irago Channel, were surveyed by dredging. The water depth was 20 to 30 meters. The smallest benthic amphioxus was14 mm and the largest one was 68 mm. The suitable sediment for habitat of Enshu-Nada Sea amphioxus has a median particle size of 0.15–1.7 mm. The maximum density of population is approximately 70 amphioxuses per square meter. The breeding season starts in June and finishes in the end of August. A winter spawning in amphioxus was reported by Chin (1941), but it has not been observed during our ecological research in Enshu-Nada Sea.

In July 2000, a natural spawning of Japanese amphioxus collected from Enshu-Nada Sea was observed at the aquarium of the Ocean Research Institute, University of Tokyo and at the Misaki Marine Biological Station, which has a nearby population of amphioxus (Nishino *et al*., 1999). The condition for inducing artificial spawning has not yet been discovered, but natural spawning in the laboratory will contribute to developmental studies of amphioxus in the future.

Amphioxus is a unique invertebrate that has an organ considered to be homologous with the vertebrate pituitary gland. The organ is called "Hatschek's pit". Several lines of evidence to support its homology with the pituitary gland have been reported as follows: the developmental similarity, observation of peptidergic granules, and immunoreactivities with human LH β (Nozaki and Gorbman, 1992) and rat Pit-1 antisera (Candiani and Pestarino, 1998). However, no chemical data have been reported for the Hatschek's pit.

I have attempted to isolate cDNAs encoding pituitary hormones through two different cloning methods. The first method is a subtraction cloning using a subtracted cDNA library between mRNA from muscles and the tissue including the pit and wheel organ. Second is an immunoscreening of an expression cDNA library constructed from the pit and the surrounding tissues. At present, 106 cDNA clones isolated from the subtracted cDNA library showed homologies to registered amino acid sequences, out of which three clones are homologues of insulin-like peptide, IGF binding protein and inhibin, respectively. An immuno-reactive clone to human LH β antiserum obtained from the expression cDNA library encodes amino acid sequence similar to a subunit of GTP binding protein isoform. No cDNA clone encoding an amino acid sequence of pituitary hormones has been obtained yet.

Recently Gorbman (1999a, b) and Nozaki *et al.* (1999) reported an outgrowth from the nerve cord toward the Hatschek's pit along the right side of the notochord.

Secretory granules were found not only in the pit but also in this outgrowth. The agnathan pituitary develops a thick sheet of connective tissue between the neurohypophysis and the adenohypophysis (Nozaki *et al*., 1994), which is a similar configuration to the Hatschek's pit-nerve cord outgrowth complex in amphioxus. From this anatomical similarity, we hypothesize a phylogenetic relationship of these organs between Agnatha and amphioxus. If the Hatschek's pit were homologous with the vertebrate adenohypophysis, the nerve cord outgrowth could be an homologue with the neurohypophysis or the median eminence. This hypothesis should be proved by the existence of pituitary hormone genes expressed in the pit and the nerve cord in amphioxus. Many other analyses of endocrine substances and functions also should be carried out for understanding the role of the Hatschek's pit.

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Preservation of Amphioxus Eggs and Embryos

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Amphioxus has been attracting several generations of biologists owing to its key position in phylogeny and its utility as a developmental model for the ancestor of the vertebrates. It has become one of the most important experimental animals in developmental biology. Most developmental biologists working on amphioxus use its eggs and embryos as materials to study the mechanism of developmental regulation. However, it is not easy to get large amounts of eggs or embryos of this animal when needed. Its special spawning behaviour and restricted breeding season make the ralated research work inconvenient. In order to solve this problem, we tested methods for extending the viability period for amphioxus eggs and embryos in the summer of 2000.

During the breeding season of Qingdao amphioxus, *Branchiostoma belcheri tsingtauense*, the sexually ripe adults were collected from the bottom of coastal water in the vicinity of Qingdao and cultured in the laboratory. Eggs and embryos at various stages of development were collected and devided into testing groups. Three preservation temperatures were used in this experiment: low temperature preservation, freezing and cryopreservation. In each case, the eggs and embryos were washed with filtered sea water for 3 times before treatment.

In the low temperature preservation test, 4°C and 7°C were chosen as storing temperature and 22 groups were set up according to different developmental stages and treating times. Survival rates of 80% and 83% were obtained after treatment for 24 hr at 7°C and 4°C respectively, while they declined to 40% and 17% after treatment for 48 hr when unfertilized eggs were tested. When the embyros of 16-cell stage were stored at 4°C for 2 hr, the survival rate was about 58% which was much lower than that of unfertilized eggs stored at 4°C for 24 hr (83%) , implying that the unfertilized egg is easier to preserve than embryo. However, to store the eggs at low temperature can only extend the developmental period for several hours or a couple of days.

In the freeze preservation test, the eggs and embryos were stored at –80°C. The eggs or embryos were pretreated and underwent a freezing-thawing procedures (Yan *et al*., 1995). Two kinds of cryoprotector, DMSO and glycerin, were used to protect the cells from damage by freezing. At the 1-cell stage with DMSO as the cryoprotector, the survival rate of eggs stored at –80°C was 23%, but with glycerin as the cryoprotector the survival rate was only 17%. Even lower survival rates (DMSO: 13% and glycerin: 11%) were obtained when the 16-cell stage embryos were stored under the same conditions.

In the cryopreservation test, the eggs and embryos were stored at –196°C (in liquid nitrogen). At such a low temperature the metabolism of the cells was completely shut down. Cryopreservation has its unparalleled superiority in keeping the developmenntal potential of the frozen-thawed embryos. Vitrification process was performed following the Valdez's method (Valdez, 1985) with modification according to the osmotic pressure of sea water. Glycerin, DMSO and vitrification solution (Vsl) were used to protect the cells when stored in liquid nitrogen. Comparison of survival rate was made with these 3 reagents. At the 2-cell stage, the survival rates of eggs vitrified with glycerin, DMSO or Vsl and stored in liquid nitrogen were 33%, 31% and 37% respectively. When the blastulae were vitrified with glycerin, DMSO or Vsl and stored in liquid nitrogen, the survival rates were 36%, 27% and 38% respectively. These results showed that in cryopreservation, Vsl is the best reagent to vitrify and preserve the amphioxus eggs or embryos when they are stored in liquid nitrogen and there is no obvious change of survival rate in different developmental stages from 2-cell to blastula. After thawing it is very important to get rid of the cryoprotective reagent because a cryoprotector such as DMSO can effect developmenal ability.

Comparing the above three preservation methods, cryopreservation has its advantage in long-term preservation of the amphioxus eggs and embryos. However, if extended manipulating time is needed when experiments are performed during the spawning season, the low temperature preservation is enough for many experiments.

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Presence, Antibacterial Action and Development of Phenoloxidase in Amphioxus *Branchiostoma belcheri tsingtauense*

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Because amphioxus has no cellular components in the blood flow, the immune or host defense system is an interesting subject. We have studied an enzyme, which is a possible player in host defense system in amphioxus.

Phenoloxidase (PO) or tyrosinase (EC1. 14. 18. 1) is a bifunctional copper-dependent enzyme, which catalyses the conversion of phenolic substances such as dihydroyphenylalanine (L-dopa) to dopaquinone, followed by several intermediate steps that lead to the synthesis of melanin, a brown pigment. PO has been shown to be present in both invertebrates and vertebrates, and considered as a putative molecule active in the immune response (Cicero *et al.*, 1982; Johansson and Soderhall, 1996). However, little is known about PO in amphioxus. In histochemical analysis, it was shown that PO activity existed in epidermal cells and endodermal epithelial cells of the gill bars and in the intestine of adult amphioxus, which was detected as brown melanin deposits. No deposits were found in the nerve cord, notochord, muscles and fin-boxes. Ultrastructural examination revealed that PO reaction products were observed as cytoplasmic granules in the epidermis and in the endodermal epithelium of the gill bar and intestine. They were electron-dense and homogenous. Frequently, PO reaction products were also observed within the secondary lysosomes.

Development of tyrosinase has been investigated only in amphibian embryos, where it is first synthesized at the early neurula stage immediately following the embryonic induction of the neural plate by the underlying chordamesoderm (Benson and Triplett, 1974). Developmental change in phenoloxidase activity in amphioxus was also studied biochemically and histochemically. PO activity initially appeared in the ectoderm including the neural ectoderm and the epidermis at the early neurula stage but not in the mesoderm or the endoderm; PO activity disappeared in the neuroepithelium but remained unchanged in the epidermis when the neural plate became detached from the rest of the ectoderm. PO could serve as a marker enzyme for differentiation of the neural ectoderm from the epidermal ectoderm during embryonic development of amphioxus.

Phenoloxidase is not only found intracellularly in blood cells of vertebrates and invertebrates, but also extracellularly in plasma without cells and in the cuticle of some insects. PO is present in an inactive form, prophenoloxidase (proPO), and activated by proteases, organic solvents or detergents (Ashida and Soderhall, 1984). In arthropods, PO is involved in the process of sclerotization and wound-healing of the cuticle as well as in defense mechanisms participating in encapsulation and melanisation against invading microorganisms (Sugumaran, 1996; Li *et al.*, 1992). The prophenoloxidase activating system is a complex cascade system mediating recognition and directing blood cell activity in a complement-like manner. Therefore, the PO and proPO activating system seem to be important factors in at least the humoral immunity system of crustaceans and insects. Biochemical detection demonstrated the presence of relatively low PO activity in the mucus obtained from the skin of amphioxus. When the mucus was pretreated with activators such as SDS, trypsin or zymosan, however, its PO activity increased significantly, indicating that the PO activity existed in the mucus predominantly as an inactive precursor, prophenoloxidase. The proPO in amphioxus, like that of insects and crustaceans, is readily activated by activators. This shows that they share some common properties. PO activity in the mucus was inhibited by phenylthiourea (PTU), a specifi inhibitor of PO. The mucus served as protecting agents against environmental pathogens and foreign bodies. Although the mucus had little antibacterial activity against *E.coli* by itself, it enhanced the antibacterial activity of L-dopa. Moreover, the enhancement of the antibacterial activity of L-dopa by the mucus was markedly suppressed by addition of PTU. These results strongly suggest that it is the PO that functions as a defense factor against invading materials in the body surface which may contain dopa.

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